Differential response of soybean (Glycine max (L.) Merr.) genotypes to lipo-chito-oligosaccharide Nod Bj V (C_{18:1} MeFuc)

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Abstract

Lipo-chito-oligosaccharides (LCOs) are bacteria-to-plant signal molecules essential for the establishment of rhizobia-legume symbioses. LCOs invoke a number of physiological changes in the host plants, such as root hair deformation, cortical cell division and ontogeny of complete nodule structures. The responses of five soybean cultivars to Nod Bj V (C_{18:1} MeFuc) isolated from Bradyrhizobium japonicum strain 532C were studied with a new technique. Two distinct types of root hair deformation were evident (i) bulging, in which root hairs were swollen at the tip or at the base depending on the cultivars and (ii) curling. The nodulating capacity of B. japonicum 532C varied among cultivars. Cultivars that produced a bulging reaction when treated with LCO had fewer nodules and the roots had low phenol contents. Cultivars that produced curling had higher numbers of nodules and the roots had higher amounts of phenol. Further, the roots of cultivars that showed root hair bulging were able to degrade LCO much faster than cultivars that manifested curling. The results of the present study establish relationships among the type of LCO-induced root hair deformation, root system LCO-degrading ability and nodulation capacity of soybean cultivars.

Key words: Nod factor, lipo-chito-oligosaccharide, soybean, root hair deformation, nodulation, phenol.

Introduction

Bacteria of the genera Rhizobium, Bradyrhizobium, Sinorhizobium, and Azorhizobium, collectively known as rhizobia, form specialized organs called nodules on the roots and sometimes stems of legumes and fix atmospheric nitrogen within these structures. Nodule formation is a highly specialized process that requires cross ‘talk’ between the bacteria and the host plant. In general, the interaction is a two-step process. The first is the release of the plant-to-bacteria signal molecules, usually specific flavonoids, by the host plants. The second step is the release of bacteria-to-plant signal molecules, which are lipo-chito-oligosaccharides, so-called nod factors (Long, 1989; Kondo, 1991; Schenes et al., 1990; Boone et al., 1999). Lipo-chito-oligosaccharides are known to affect a number of host plant physiological processes. For example, they induce root hair deformation (Spaink et al., 1991), ontogeny of compete nodule structures (Fisher and Long, 1992; Denarie and Cullimore, 1993), cortical cell division (Sanjuan et al., 1992; Schulman et al., 1997), and the expression of host nodulin genes essential for infection thread formation (Horvath et al., 1993; Pichon et al., 1993, Minami et al., 1996). LCOs have also been shown to activate defence-related enzymes (Inui et al., 1997). Further, a number of studies have shown that the structure of LCOs appears to modulate the host specificity of rhizobia–legume interactions (Spaink et al., 1991; Schultze et al., 1992).

Biological assay is an important step in the isolation of LCOs. A number of assay systems are available, such as depolarization of root hair membranes in alfalfa (Ehrhardt et al., 1992), and induction of ENOD12 gene expression in the root epidermis of alfalfa (Pichon et al., 1993). However, the root hair deformation assay was one of the earliest and the most commonly used bioassays for LCOs. The most commonly used method for root hair deformation assay is the Fahraeus slide technique (Fahraeus, 1957) and modified Fahraeus slide technique.
(Heidstra et al., 1994). In this technique seeds are grown with roots clamped between a glass slide and a coverslip and the whole set-up is placed in the test solution; root hair deformation is observed with an inverted light microscope. However, this technique cannot be used to study large-seeded legumes such as soybean and bean due to the thickness of their roots.

The root hair deformation activity of LCOs has been studied in a number of legumes (Heidstra et al., 1994; Relic et al., 1993). Different types of root hair deformation have been reported, eg., branching and curling. The mechanisms by which the LCO elicits root hair deformation have been the subject of investigation and several plausible explanations have been suggested. It has been postulated that alteration in root hair structure might be due to the reinitiation of root hair growth (Heidstra et al., 1994) and noted that LCOs cause a rapid and transient change in ion fluxes, particularly Ca$^{2+}$ (Felle et al., 1999). Such changes in the cytosol of the root hair apex could lead to root hair deformation. However, the types of root hair deformation elicited by LCOs in different genotypes of soybean (Glycine max) and its correlation with nodulation patterns has not been explored.

Root chinatines are known to degrade LCOs (Heidstra et al., 1994; Xie et al., 1999). The potential of chinatines to degrade LCOs has been projected to account, at least in part, for host specificity, complementing the role of LCO structural variability (Staehelin et al. 1994). Recently, differences were reported in the chinatine activity in the roots and nodules of five different genotypes of soybean (Xie et al., 1999). The increase in the chinatine activity was dependent on the host-bacteria combinations and there was no relationship between chinatine activity and nodule number.

This paper describes a simple technique to study the effect of LCO on root hairs of a large-seeded legume, soybean. Using this technique, the differential responses of five cultivars of soybean to LCO isolated from genistein-induced cultures of Bradyrhizobium japonicum 532C were investigated. A correlation has been established between the root hair responses and LCO-degrading potential of different genotypes of soybean and nodulation patterns and levels.

**Materials and methods**

*Isolation and purification of lipo-chito-oligosaccharide from Bradyrhizobium japonicum strain 532C*

Bradyrhizobium japonicum (strain 532C) was grown at 28 °C in yeast mannitol medium (YEM) (mannitol 10 g, K$_2$HPO$_4$ 0.5 g, MgSO$_4$·7H$_2$O 0.2 g, NaCl 0.1 g, yeast extract 0.4 g, and distilled water 1000 ml), pH 6.8, shaken at 150 rpm until the OD$_{620}$ reached 0.4–0.6 (4–6 d) in the dark. Thereafter, 2.0 l of bacterial subculture was started by inoculating material from the first culture (5 ml of the first culture per 250 ml of YEM media), for 5–7 d (OD$_{620}$ 0.8–1.0), as above. At this stage, 0.25 ml of 50 μM genistein (in methanol) was added to each 250 ml of bacterial subculture (genistein concentration of 5 μM) and the culture was incubated for 48–96 h.

Two litres of bacterial subculture along with the bacterial biomass were phase-partitioned against 0.8 l of HPLC-grade 1-butanol by shaking overnight. The upper butanol layer was transferred to a 1.0 l evaporation flask and concentrated to 2–3 ml of light brown, viscose material with a rotary evaporator operated at 80 °C (Yamata RE500, Yamato, USA). This extract was resuspended in 4 ml of 18% acetonitrile and kept in the dark at 4 °C in a sealed glass vial until use.

HPLC (equipped with Waters Model 510 HPLC pump, Waters model 712 WISP and Waters model 410 differential refractometer. Waters, MA, USA) analysis was conducted with a Vydis C18 reversed-phase column (Vydis, CA, USA; catalogue no. 218TP54) with a flow rate of 1.0 ml min$^{-1}$ and a Vydis guard column (catalogue no. 218GK54). As a baseline 18% acetonitrile (AcN/H$_2$O; w/w) was run through the system for at least 10 min prior to injection. The sample was loaded and isocratic elution was conducted with 18% of AcN for 45 min to remove all non-polar light fractions. Thereafter, gradient elution was conducted for 90 min with 18–82% AcN. The LCO eluted at 94–96 min of HPLC run time.

**Root hair deformation assay**

Seeds of soybean (cultivars: AC Bravor, OAC Brussels, Maple Glen, Nordet, cv. 9007) were surface-sterilized with 2% sodium hypochlorite for 2 min and washed with at least four changes of sterile distilled water. The seeds were then placed on 1.5% water agar (20 ml) in 9 cm diameter Petri dishes (two seeds per plate). The Petri dishes were incubated in the dark at 25 °C for 7–8 d; during this time the seeds germinated and developed tap and lateral roots on the agar surface. Lateral roots with abundant root hairs, which could be easily distinguished by the fluffy growth, were excised with a sterile scalpel. These lateral roots were placed on sterile grease-free glass slides containing 40–60 μl of LCO solution. The slides were then placed in a closed moist chamber and incubated for different periods (6, 24 h) at 25 °C in the dark. At the end of the incubation time the slides were removed and the roots were fixed in a staining solution [methylene blue (0.02% w/v)+glicerol (20% v/v)+phenol (10% w/v)]. Slides were observed under a light microscope for root hair deformation. Each treatment had at least three replicate lateral roots, and a minimum of 100 root hairs was observed from each replicate. The root hair deformation was categorized into two types: bulging or curling. The percentage deformation in each category was calculated. Root segments about 1–2 mm above the tip showed the maximum deformation and so this area was utilized.

**Nodulation experiment**

Soybean seeds (same cultivars as in the previous work) were surface-sterilized as described above. The seeds were then planted in trays containing a mixture of surface (an inert calcined clay. Applied industrial Materials Corp., Illinois, USA) and sand (3:1). Seedlings at the vegetative cotyledonary (VC) stage (Fehr et al., 1971), about 7–10-d-old, were transplanted into plastic pots (13 cm diameter) containing the same medium. The seedlings were inoculated with 1 ml of Bradyrhizobium japonicum 532C grown in yeast extract mannitol broth for 7 d, cell density adjusted to 10$^8$ cells ml$^{-1}$ (OD$_{620}$=0.08) with distilled water. The plants were grown in a glasshouse...
maintained at 25±2 °C with a 14:10 h day/night cycle with supplemental light from high pressure sodium lamps (800–1200 µmol m⁻² s⁻¹). After 30 d the plants were harvested, taking care not to disturb the root system. The roots were washed with tap water and the number of nodules counted. The nodules were then dried at 90 °C for 24 h and the dry weight taken. There were at least five replicates for each cultivar.

Degradation of LCO by roots of soybean

Soybean seeds (cultivars: Maple Glen and AC Bravor) were surface-sterilized with 2% sodium hypochlorite for 2 min, washed with four changes of sterile distilled water and germinated in plastic trays containing a bed of autoclaved vermiculite. After 8 d they were carefully uprooted and the roots were washed gently in running water. Plants with 5 cm² of roots were placed in 120 ml of 10⁻³ M solution of LCO in plastic containers located on a glasshouse growth bench maintained at 25±2 °C with a 14/10 h day/night cycle with supplemental light from high pressure sodium lamps (800–1200 µmol m⁻² s⁻¹). The plant roots remained in the solution for 24 h. The solution in each container was aerated by bubbling with compressed air (650–700 cm³ min⁻¹). There were three replicates of each cultivar. Plastic containers with LCO solution, but without plants, served as controls. After 24 h the contents of the container were stirred and 25 ml was pipetted into test tubes and freeze-dried. The freeze-dried material was dissolved in 1 ml of 18% acetonitrile and analysed with HPLC for the concentration of LCO as described in the LCO purification section.

Estimation of root phenol content

Plants were raised as described immediately above. After 7 d, the plants were uprooted and 1 g of fresh roots was removed and washed in distilled water. Free phenol was estimated as described previously (Bray and Thorpe, 1954).

Data analysis

The data were analysed for statistical significance by an ANOVA protected LSD test using SAS (Statistical Analysis System) (SAS Institute Inc., 1989).

Results and discussion

Lipo-chito-oligosaccharide could be isolated from the genistin-induced cultures of *B. japonicum* strain 532C as an HPLC peak with the same retention time as that of the LCO of strain USDA 110 (standard provided by G Stacey, University of Tennessee). The LCO peak isolated from *B. japonicum* strain 532C showed the correct biological activity in that it caused soybean root hair deformation. The structure was confirmed by MS/MS analysis (RW Carlson, Complex Carbohydrate Research Center, University of Georgia, Athens) and was found to be the major LCO of *B. japonicum* with the nomenclature Nod *Bj* V (C₁₈:1; MeFuc). Root hair deformation could be observed over a range of LCO concentrations (10⁻⁶–10⁻⁸ M) in all the cultivars of soybean used in this study. In general, the percentage of deformation increased with concentration and the time of incubation (Tables 1, 2). For the cultivar AC Bravor the deformation could be observed as early as 3 h after treatment (results not shown). Root hairs of soybean cultivars

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>10⁻⁶ M</th>
<th>10⁻⁷ M</th>
<th>10⁻⁸ M</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Curling</td>
<td>Bulging</td>
<td>Curling</td>
</tr>
<tr>
<td>AC Bravor</td>
<td>53 ± 2.5</td>
<td>0</td>
<td>43 ± 2.3</td>
</tr>
<tr>
<td>OAC Brussels</td>
<td>15 ± 1.6</td>
<td>0</td>
<td>6.3 ± 1.8</td>
</tr>
<tr>
<td>Maple Glen</td>
<td>0</td>
<td>3.3 ± 1.9</td>
<td>0</td>
</tr>
<tr>
<td>Nordet</td>
<td>2.6 ± 0.3</td>
<td>40.6 ± 1.0</td>
<td>3 ± 0.8</td>
</tr>
<tr>
<td>cv. 9007</td>
<td>9 ± 0.5</td>
<td>1 ± 0.5</td>
<td>11 ± 1.2</td>
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</table>

Values are the mean of three replicate lateral roots ± standard error.

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>10⁻⁶ M</th>
<th>10⁻⁷ M</th>
<th>10⁻⁸ M</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Curling</td>
<td>Bulging</td>
<td>Curling</td>
</tr>
<tr>
<td>AC Bravor</td>
<td>80 ± 1.6</td>
<td>0</td>
<td>54 ± 2.3</td>
</tr>
<tr>
<td>OAC Brussels</td>
<td>73.6 ± 3.0</td>
<td>0</td>
<td>62.3 ± 3.5</td>
</tr>
<tr>
<td>Maple Glen</td>
<td>5 ± 3.3</td>
<td>57.6 ± 0.8</td>
<td>0</td>
</tr>
<tr>
<td>Nordet</td>
<td>0</td>
<td>47.3 ± 5.3</td>
<td>0</td>
</tr>
<tr>
<td>cv. 9007</td>
<td>37 ± 2.5</td>
<td>3.3 ± 1</td>
<td>28 ± 2.6</td>
</tr>
</tbody>
</table>

Values are the mean of three replicate lateral roots ± standard error.
responded differentially to the LCO treatment and two distinct types of reaction were evident. Bulging consisted of considerable swelling [up to 2–3 times the diameter of untreated controls (Fig. 1A)] of the root hairs either at the base, as was the case in cv. Nordet (Fig. 1B), or at the tip in the case of cv. Maple Glen (Fig. 1C). Curling varied from moderate to extreme curling, like a ‘shepherd’s crook’, and was most common in AC Bravor (Figs 1D, E).

Cultivars differed in the time required to respond to LCO treatment (Tables 1, 2). At 6 h after treatment AC Bravor showed the maximum root hair deformation (53%) at the highest LCO concentration of 10⁻⁶ M, followed by Nordet in which 40.6% of the root hairs were deformed while in other cultivars 1–25% deformation was observed. After 24 h three of the cultivars had more than 60% root hair deformation due to LCO treatment.

Roots of soybean cultivars differed in their potential to degrade LCO. Two cultivars, AC Bravor (typical curling of root hairs in response to LCO) and Maple Glen (bulging response) were tested for their potential to degrade LCO. Roots of the cv. Maple Glen degraded 96% of the LCO over 24 h as compared to AC Bravor, which degraded only 60% of the LCO in the medium.

Only 47% of the initial LCO could be recovered in the control that did not have any roots. This may have been due to adsorption of the lipid portion of the LCOs to the walls of the plastic containers (G Stacey, personal communication). All the values presented were, therefore, corrected against the control.

The phenol content of the root systems was measured because the plant-to-bacteria signal molecules are phenolic compounds (largely daidzein and genistein in soybean), and previous work has shown that under some conditions the levels of these compounds can be limiting to the nodulation process (Zhang and Smith, 1995). Cultivar AC Bravor had the greatest phenol content of the cultivars utilized in this study, while Nordet had the lowest (Fig. 2). Thus, a cultivar that responds quickly and ‘normally’ (i.e. root hair curling) to LCOs had more phenol in its roots, and therefore had higher levels of at least parts of the pathway leading to the plant-to-bacteria signals, while a cultivar that rapidly produced root hair bulging had lower levels.

*Bradyrhizobium japonicum* 532C was able to nodulate all the soybean cultivars used in this study (Fig. 3). However, the nodulation response varied among the cultivars. OAC Brussels had the largest number of nodules followed by cv. 9007

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![Fig. 1. Root hair deformation induced by the lipo-chito-oligosaccharide Nod Bj V C18:1 MeFuc. Control showing no root hair deformation (A). Bulging of root hair tips observed in the cultivar Maple Glen (B). Bulging of the base of root hairs in Nordet (C). Wiggling (D) and typical root hair curling (E) in AC Bravor.](image-url)
and AC Bravor. Cultivars Nordet and Maple Glen had significantly fewer nodules. Thus low nodulation corresponds to low phenol content and root hair swelling.

The excised lateral root technique described in this paper is simple and reliable and offers the following advantages: (1) large-seeded legumes can be investigated in a relatively easy manner; (2) the method is rapid and can be used for large-scale screening of potentially effective compounds as roots from a single plant could be used to test the activity of a number of compounds, and (3) the method requires very little of the test solutions (40–50 µl).

There were differences in the susceptibility of the cultivars to root hair deformation induced by LCO. It was observed that the deformation in vetch and alfalfa roots started within 1 h of LCO treatment and typical deformation could be visualized at the end of 3 h (Heidstra et al., 1994). In the present study AC Bravor responded earlier than the other cultivars. Although there are number of reports on root hair deformation induced by LCO (Heidstra et al., 1994; Relic et al., 1993), to the authors’ knowledge this is the first report of the effect of an LCO on different genotypes within the same species. The implications of the differential response of genotype to LCO warrants further investigation. However, it is worth while noting that the results from this laboratory have shown that treatment of soybean with genistein increased nodulation and yield under controlled environment and field conditions (Zhang and Smith, 1996; Dashti et al., 2000). AC Bravor, which responded earlier to LCO and with curling was more responsive than Maple Glen, a cultivar that was slower to respond to LCO and typically responded with bulging. The differences in the reaction types probably represent aspects of strain—cultivar specificity. Numerous studies have shown that only certain strains of rhizobia can effectively nodulate a given genotype of a legume (Xie et al., 1999).

Plant chitinases are known to hydrolyse LCOs and chitinases from different plant species varied in their ability to degrade LCOs (Staehelin et al., 1994).
A symbiosis-related chitinase isozyme was induced in response to *B. japonicum* and nod factors (Xie et al., 1999) suggesting that these isozymes might be important in the early signal transduction process of nodulation. In the present study cultivars varied in their ability to degrade LCO. It is interesting to note that the cv. Maple Glen, which showed a bulging reaction to LCO, was able to degrade LCO faster than other cultivars and formed fewer nodules, while AC Bravor which was less effective in degrading LCO, manifested a curling response to LCOs and formed more nodules. These results suggest that the root hair response to LCO and the LCO hydrolysing capacity of roots might potentially be used as indicators of nodulation capacity. It is possible that genotypes which have less potential for nodulation react hypersensitively to LCO resulting in production of chitinases and other hydrolysing enzymes causing rapid degradation of LCOs and that this interaction results in bulging of root hairs.

**Acknowledgements**

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